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Two glucosylceramide synthase inhibitors attenuate doxorubicin-induced p21$^{Cip1/Waf1}$ upregulation in HepG2 cells, irrespective of their differential chemosensitizing properties

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Abstract

We have previously reported that HepG2 human hepatocarcinoma cells are sensitized to doxorubicin-induced apoptosis by the glucosylceramide synthase inhibitor D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) but not by the more specific inhibitor D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP). Herein we investigated whether the chemosensitizing action of PDMP impinged on any unspecific effect of this compound on doxorubicin-induced expression of p53 and/or p21$^{Cip1/Waf1}$, namely two proteins reported to modulate the apoptotic response to DNA-damaging agents, in a positive or negative fashion, respectively. We show that, in HepG2 cells, PDMP did not substantially affect doxorubicin-induced p53 upregulation, whereas drug-evoked upregulation of p21$^{Cip1/Waf1}$ was markedly attenuated. Although this outcome could be expected to account for the chemosensitizing effect of PDMP, impaired upregulation of p21$^{Cip1/Waf1}$, in the setting of unaltered p53 expression, was also observed in the case of PPPP. These results, while raising the possibility of a link between attenuation of drug-evoked p21$^{Cip1/Waf1}$ expression and redirection of (glyco)sphingolipid metabolism, show that, differently from other tumor systems, attenuation of doxorubicin-induced p21$^{Cip1/Waf1}$ expression is at least not sufficient to sensitize HepG2 cells to the apoptotic action of the drug.

Keywords: Hepatocarcinoma; Doxorubicin; Apoptosis; p21$^{Cip1/Waf1}$; p53; Glucosylceramide synthase inhibitor

Increasing evidence indicates that the cyclin-dependent kinase inhibitor p21$^{Cip1/Waf1}$ is a multi-functional protein, controlling both cell cycle progression and apoptosis [1–3]. The expression of p21$^{Cip1/Waf1}$ is under the control of several transcription factors, most notably p53, a protein also displaying pro-apoptotic properties, that is, in turn, upregulated in response to cell treatment with DNA-damaging agents [3]. Following cell exposure to genotoxic drugs, p21$^{Cip1/Waf1}$ may be upregulated in a p53-dependent fashion, thus inducing cell cycle arrest to allow DNA repair. In this context, however, it has also been reported to fulfill a relevant anti-apoptotic function: in fact, studies on various tumor systems, including breast, prostate, and colon cancer cells, indicate that attenuation or loss of p21$^{Cip1/Waf1}$ function provides a favorable setting for apoptosis induction [4–6]. Indeed, in consideration of the pro-apoptotic activity of p53, it has also been proposed that the relative expression of p53 to p21$^{Cip1/Waf1}$ may provide a key factor dictating cell response to genotoxic agents in terms of arrest of the replicative cycle or apoptosis induction [3].

HepG2 cells provide a well-established human hepatocarcinoma system harboring functional p53 and

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$p21^{Cip1/Waf1}$ [7,8]. A study from this laboratory has shown that HepG2 cells are scarcely sensitive to the apoptotic action of doxorubicin, used at pharmacologically relevant doses, but can be sensitized to the cytotoxic activity of the drug by co-treatment with the glucosylceramide synthase (GCS) inhibitor D,L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) [9]. It is generally assumed that GCS inhibitors exert their chemosensitizing effect by potentiation of drug-induced intracellular ceramide elevation [10]. Nevertheless, we also reported that the chemosensitizing effect observed with PDMP was not reproduced by the more specific GCS inhibitor D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), although these compounds were used at equipotent concentrations and both enhanced doxorubicin-induced ceramide accumulation [9]. These findings strongly suggested that PDMP relieves a downstream restraint in the apoptotic pathway triggered by doxorubicin independently from GCS inhibition, a notion indeed corroborated by observations on other cell systems [11]. On this basis, we decided to investigate whether the chemosensitizing action of PDMP, in HepG2 cells exposed to doxorubicin, impinged on any unspecific effect of this compound on drug-induced $p21^{Cip1/Waf1}$ and/or p53 expression.

Materials and methods

Materials. Material for cell culture, doxorubicin, and the anti-β-actin mouse monoclonal antibody were from Sigma Chemical (St. Louis, MO, USA). PDMP and PPPP were from Calbiochem–Novachem (La Jolla, CA, USA). The anti-$p21^{Cip1/Waf1}$ rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-p53 mouse monoclonal antibody was from Upstate Biotechnology (Lake Placid, New York, USA). The chemiluminescence ECL detection system was from Amersham (Bucks., UK).

Cell culture and apoptosis evaluation by flow cytometric analysis. Cells were grown at 37 °C in RPMI-1640 medium supplemented with 10%(v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere with 5%(v/v) CO_2. Cell treatments were performed in complete growth medium. Doxorubicin was administered from a 1 mg/ml stock solution in RPMI-1640 medium. Stock solutions of PDMP (30 mM) and PPPP (1 mM) were prepared in dimethyl sulfoxide. Apoptosis was monitored by evaluation of the hypodiploid cell population after flow cytometric analysis, as previously reported [12]. Briefly, cells were detached from the plates by trypsin treatment, washed with phosphate-buffered saline (pH 7.4), and resuspended in 0.5 ml of a solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Cells were left at 4 °C for 30 min at least, in the absence of light, and analyzed using a FACScan Flow Cytometer (Becton–Dickinson, CA); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed by the Lysis II program (Becton–Dickinson, CA).

Western blotting. Cells were washed with phosphate-buffered saline (pH 7.4) and lysed in 62 mM Tris–HCl, pH 6.8, containing 2% SDS, 2 mM EDTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate. After sonication, aliquots of lysates were saved for protein determination [13] and, after addition of 0.05% β-mercaptoethanol, samples were boiled for 10 min. Proteins from cell lysates (40 μg) were resolved by SDS–PAGE, transferred overnight at 25 mA onto nitrocellulose paper, and analyzed by Western blot as previously described [14]. ECL detection was performed according to the manufacturer's instructions. The densitometric analysis of blots was performed by a computerized image processing system (Gel-Pro Analyzer).

Results

Fig. 1 shows a time-course of the apoptotic response evoked by HepG2 cell treatment with doxorubicin (1 μg/ml), employed either alone or in combination with a PDMP concentration (30 μM) previously reported to inhibit by about 90% glucosylceramide synthesis, in intact cells [9]. It can be observed that doxorubicin, administered alone, was scarcely toxic to HepG2 cells, evoking a moderate apoptotic response only by 48 h. PDMP, administered alone at the above-reported concentration, was not toxic to HepG2 cells, at least over a 48-h period (not shown); however, when co-administered with doxorubicin, it dramatically accelerated and propagated drug-induced apoptosis. As shown in Fig. 1, cell death induced by doxorubicin plus PDMP was at its onset already by 24 h and by 48 h over a 70% of the total cell population was found apoptotic. Under the experimental conditions described above, $p21^{Cip1/Waf1}$ expression was also monitored. Figs. 2A and C show that, after administration of doxorubicin alone, $p21^{Cip1/Waf1}$ levels were dramatically upregulated, in a time-dependent fashion, from the beginning of incubation up to 24 h, remaining fairly constant from this time to 48 h.

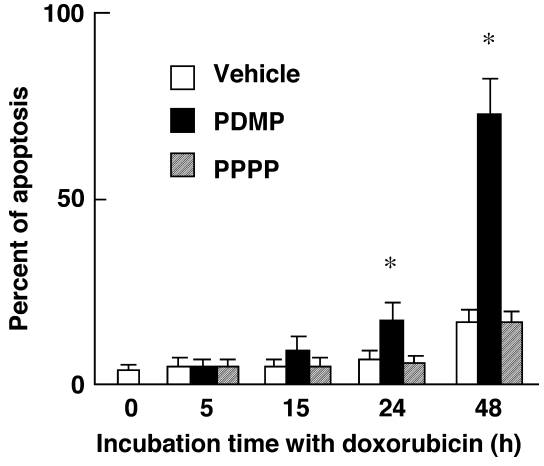

Fig. 1. Time-course of the apoptotic effect of doxorubicin, administered to HepG2 cells either alone or in combination with PDMP or PPPP. Cells were preincubated for 1 h with vehicle (0.1% DMSO), or with 30 μM PDMP or 1 μM PPPP and then exposed to 1 μg/ml doxorubicin. At the indicated times, cells were harvested and apoptosis was monitored by flow cytometry, as described in the text. Data are means ± SD of four different experiments. Statistical significance: *P, 0.01, as from Student's t test, in comparison with samples incubated with doxorubicin alone.

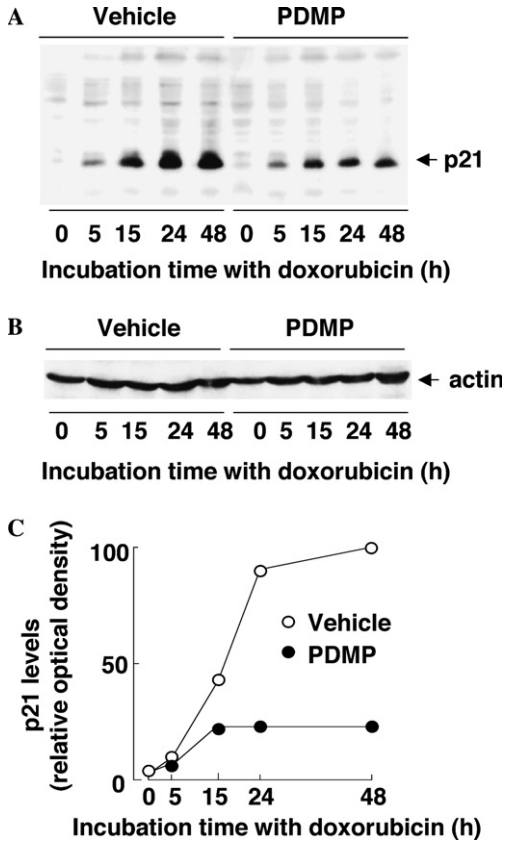

Fig. 2. PDMP inhibits doxorubicin-induced $p21^{Cip1/Waf1}$ upregulation. Cells were preincubated for 1 h with vehicle (0.1% DMSO) or with 30 μM PDMP and then exposed to 1 μg/ml doxorubicin. At the indicated times, cells were lysed and 40 μg of total proteins from each sample was analyzed by Western blotting for $p21^{Cip1/Waf1}$ (A) or for β-actin (B), to monitor loading of equal protein amounts. The relative optical densities of the $p21^{Cip1/Waf1}$ bands shown in (A) are reported in (C). Results are representative of four independent experiments.

Remarkably, in the presence of PDMP, doxorubicin-induced upregulation of $p21^{Cip1/Waf1}$ was markedly attenuated; protein levels, in fact, modestly increased from the beginning of the treatment to 15 h, remaining fairly constant as the incubation was prolonged to 48 h. It is worth noting that attenuation of doxorubicin-induced $p21^{Cip1/Waf1}$ upregulation did not appear to be a trivial consequence of acceleration and propagation of the apoptotic response. PDMP, in fact, attenuated $p21^{Cip1/Waf1}$ expression by a 50% at 15 h and by a 75% at 24 h, namely two time-points at which apoptosis potentiation by the GCS inhibitor was either not yet observed or was observed to occur only to a very low extent (compare Figs. 1 and 2C).

As shown in Fig. 2A, impaired upregulation of $p21^{Cip1/Waf1}$ was not accompanied by accumulation of products with MW lower than 21 kDa. Moreover, although it is well established that $p21^{Cip1/Waf1}$ may undergo degradation by the proteasome after ubiquitinylation [15,16], we did not detect accumulation of products immunoreactive to the anti-$p21^{Cip1/Waf1}$ antibody with MW higher than 21 kDa. These results strongly suggest that the effect of PDMP on $p21^{Cip1/Waf1}$ expression might be due to a decreased rate of protein synthesis rather than accelerated degradation.

Since $p21^{Cip1/Waf1}$ transcription is under the control of p53, that is itself upregulated in response to cell treatment with genotoxic agents, we monitored whether PDMP had any effect on doxorubicin-induced p53 expression. Fig. 3A shows that, after cell exposure to doxorubicin alone, p53 was already upregulated by 5 h; thereafter, protein levels further increased up to 24 h, to decrease only slightly from this time to 48 h. Notably, the above-reported expression pattern of p53 was not substantially affected when doxorubicin was administered in the presence of PDMP (Fig. 3).

The differential effect of PDMP on doxorubicin-induced p53 and $p21^{Cip1/Waf1}$ expression was expected to provide a most favorable setting for amplification of the apoptotic response [3]. Nevertheless, evidence for the existence of a causal relationship between impaired $p21^{Cip1/Waf1}$ upregulation and the chemosensitizing effect brought about by PDMP in HepG2 cells had yet to be achieved.

As shown in Fig. 1, the high specific GCS inhibitor PPPP was ineffective in inducing HepG2 chemosensitization to doxorubicin, although used at a concentration (1 μM), inhibiting by about 90% ceramide glucosylation, as observed for 30 μM PDMP [9]. Thus, if impaired $p21^{Cip1/Waf1}$ upregulation was central to the chemosensitizing effect of PDMP, the phenomenon had to be unspecific (i.e., not due to GCS inhibition) and, hence, not reproduced by PPPP. Surprisingly, in this respect,

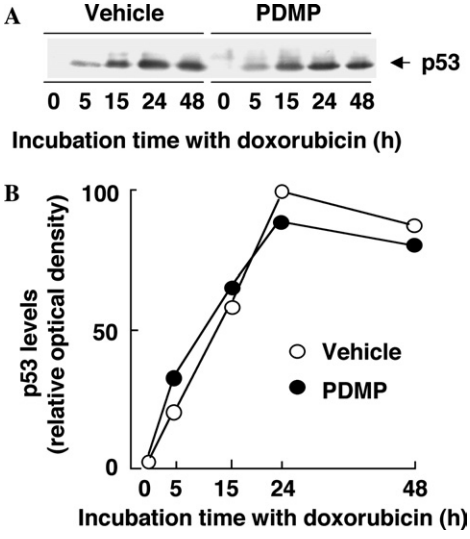

Fig. 3. PDMP does not affect doxorubicin-induced p53 upregulation. Cells were preincubated for 1 h with vehicle (0.1% DMSO) or with 30 μM PDMP and then exposed to 1 μg/ml doxorubicin. At the indicated times, cells were lysed and 40 μg of total proteins from each sample was analyzed by Western blotting for p53 (A). The relative optical densities of the p53 bands shown in (A) are reported in (B). Results are representative of four independent experiments.

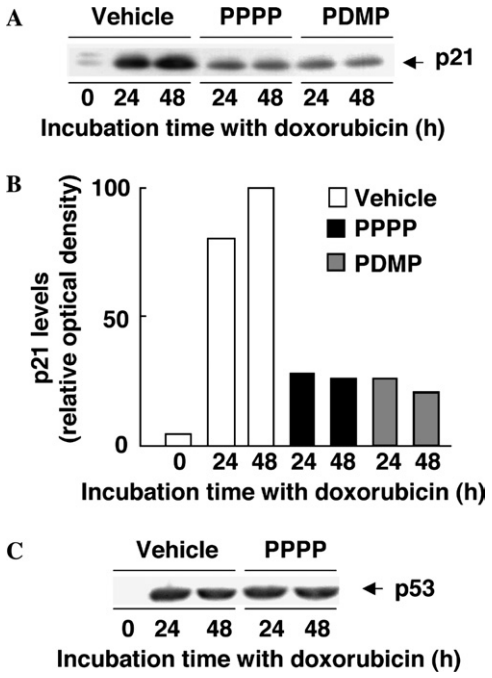

Fig. 4. PPPP inhibits doxorubicin-induced $p21^{Cip1/Waf1}$ upregulation but does not affect drug-induced p53 expression. (A) Equal amounts of proteins from lysates of untreated cells (time 0) or cells treated with 1 μg/ml doxorubicin, either alone (vehicle) or in the presence of 30 μM PDMP or 1 μM PPPP, for 24 or 48 h, were analyzed by Western blotting for $p21^{Cip1/Waf1}$. The relative densities of the bands shown in (A) are reported in (B). Results are representative of four independent experiments. (C) Equal amounts of proteins from lysates of untreated cells (time 0) or cells treated with 1 μg/ml doxorubicin, either alone (vehicle) or in the presence of 1 μM PPPP, for 24 or 48 h, were analyzed by Western blotting for p53.

we observed that PPPP was able to attenuate doxorubicin-induced $p21^{Cip1/Waf1}$ upregulation to an extent similar to that observed for PDMP (Figs. 4A and B). Moreover, similar to PDMP, PPPP did not impair drug-evoked p53 upregulation (Fig. 4C).

Discussion

The finding that both PDMP and PPPP attenuate doxorubicin-induced $p21^{Cip1/Waf1}$ upregulation in HepG2 cells, irrespective of their differential chemosensitizing effect, has two major implications. On the one hand, since, to our knowledge, effects unrelated to GCS inhibition have not been reported for PPPP, our results raise the possibility of a link between attenuation of drug-evoked $p21^{Cip1/Waf1}$ expression and redirection of (glyco)sphingolipid metabolism; in addition, pertinently to the aim of this study, data herein reported support the view that attenuation of doxorubicin-induced $p21^{Cip1/Waf1}$ expression is at least not sufficient to sensitize HepG2 cells to the apoptotic action of the drug. Thus, our results are at variance with data reported for other tumor systems, in which loss or attenuation of $p21^{Cip1/Waf1}$ function, in the setting of p53 expression, has been shown to favor apoptosis induction [4–6].

Since it has been proposed that $p21^{Cip1/Waf1}$ might block the apoptotic activity of p53, our results also cast doubts about the key role for p53 in mediating apoptosis induced by doxorubicin in HepG2 cells. In this respect, it has been shown that certain hepatocarcinoma cells lacking functional p53 nevertheless undergo apoptosis after doxorubicin treatment [17]. On this basis, one could envisage the possibility that the apoptotic action of the drug, as occurring in hepatocarcinomas, does not generally impact on p53 function.

Notably, recent studies have shown that $p21^{Cip1/Waf1}$ downregulation is a permissive event for apoptosis execution in HepG2 cells exposed to FAS-ligand or interferon-γ [18,19]; in the light of these reports, our results indicate that, even in the same cell system, the permissive effect of $p21^{Cip1/Waf1}$ downregulation on apoptosis propagation may depend on the apoptotic stimulus delivered. The reasons underlying this phenomenon remain to be elucidated; however, one possibility might stem from the observation that, in HepG2 cells exposed to FAS-ligand, the anti-apoptotic effect of $p21^{Cip1/Waf1}$ has been related to blocking of caspase 3 activation [19]. In this respect, it is worth mentioning that apoptosis induced by interferon-γ in HepG2 cells has also been shown to involve caspase 3 activation [20], whereas we previously showed that apoptosis induced by doxorubicin and propagated by PDMP, in this cell line, occurs via a caspase3-independent pathway [9]. Altogether, these observations would point to the possibility that, in HepG2 cells, the anti-apoptotic activity of $p21^{Cip1/Waf1}$ is predominantly restricted to those forms of cell death requiring caspase 3 activation.

It has recently been proposed that $p21^{Cip1/Waf1}$ might provide a novel therapeutic target in cancer treatment and the search for pharmacological agents able to impair its expression in anti-cancer regimen has been urged [20]. In the former respect, our findings highlight the necessity of a careful assessment of the anti-apoptotic function of $p21^{Cip1/Waf1}$ in the context of a particular type of cancer and of the therapeutic agent employed. On the other hand, further studies will clarify whether the negative modulation of $p21^{Cip1/Waf1}$ levels brought about by the two GCS inhibitors is a general or a cell-type specific phenomenon. In the former case, one could envisage the possibility that these compounds might be useful for inhibition of $p21^{Cip1/Waf1}$ expression in those systems in which this protein provides a key anti-apoptotic factor.

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